APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Nov. 1988, p. 2852–2855
0099-2240/88/112852-04$02.00/0

Vol. 54, No. 11

Isolation of *Rhizobium loti* Strain-Specific DNA Sequences by Subtraction Hybridization

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Received 31 May 1988/Accepted 24 August 1988

Mixed-phase (heterogeneous) and single-phase (homogeneous) DNA subtraction-hybridization methods were used to isolate specific DNA probes for closely related *Rhizobium loti* strains. In the heterogeneous method, DNA from the prospective probe strain was repeatedly hybridized to a mixture of DNA from cross-hybridizing strains (subtracter DNA) which was immobilized on an epoxy-activated cellulose matrix. Probe strain sequences which shared homology with the matrix-bound subtracter DNA hybridized to it, leaving unique probe strain sequences in the mobile phase. In the homogeneous method, probe strain sequences were hybridized in solution to biotinylated, mercurated subtracter DNA. Biotinylated, mercurated subtracter DNA and probe strain sequences hybridized to it were removed by two-step affinity chromatography on streptavidin-agarose and thiol-Sepharose. The specificity of the sequences remaining after subtraction hybridization by both methods was assessed and compared by colony hybridization with *R. loti* strains. Both methods allowed the rapid isolation of strain-specific DNA fragments which were suitable for use as probes.

Total genomic DNA probes have previously been used to identify unmodified strains of *Bradyrhizobium* and *Rhizobium* spp. in colonies and crushed legume root nodules (3, 5). While such probes are easy to prepare, they cross-hybridize with DNA from related strains. This limits their range of applications and in particular restricts their usefulness in an important area of *Rhizobium* ecology: the study of interstrain competition for legume nodulation.

The construction of probes with the degree of specificity required to distinguish between closely related strains of rhizobia would normally involve cloning and screening of genomic libraries for small fragments of DNA with unique gene sequences, a process which is impractical even for a limited number of strains. A method which quickly removes nonunique genomic sequences would be a more attractive alternative. Scott et al. (9) reported a subtraction-hybridization method for isolating particular genes from transformed mouse cells, and Welcher et al. (10) used a single-phase subtraction-hybridization technique to isolate *Neisseria gonorrhoeae*-specific clones.

A probe containing the maximum number of genomic sequences exclusive to a given organism would conceivably offer the most sensitive detection of homologous target DNA. In this report we describe mixed-phase (heterogeneous) and single-phase (homogeneous) subtraction-hybridization methods for removing nonspecific DNA sequences from strains of *Rhizobium loti* which cross-hybridize when probed with total genomic DNA. Sequences remaining after subtraction hybridization were tested for strain specificity by colony hybridization.

MATERIALS AND METHODS

DNA probes were generated for strains of *R. loti* by two subtraction-hybridization procedures. The *R. loti* strains used were NZP2014, NZP2037, and NZP2042 and were obtained from the Department of Scientific and Industrial Research, Palmerston North, New Zealand.

DNA extraction and labeling. Total genomic DNA was extracted and purified as previously described (3), with the addition of a cesium chloride density gradient purification step (7). DNA from a prospective probe strain (probe strain DNA) was digested to completion with *Mbo*I and end-labeled to a specific activity of 10^7 to 10^8 cpm μg of DNA^{-1} with [α-^{32}P]dCTP (3,000 Ci mM^{-1}), using the Klenow fragment of DNA polymerase (1). DNA from nonhomologous strains (subtracter DNA) was fragmented by sonication (three times at 30 s on ice) in an ultrasonic disintegrator (MSE, 150 W). In the heterogeneous procedure, subtracter DNA was immobilized on an epoxy-activated cellulose matrix. In the homogeneous procedure, subtracter DNA was biotinylated and mercurated to permit subsequent removal from the liquid phase by passage through a column containing thiol-Sepharose and streptavidin-agarose.

Immobilization of DNA on cellulose. Subtracter DNA was immobilized on epoxy-activated cellulose as follows. Fifty milligrams of epoxy-activated cellulose (GIBCO Ltd., Paisley, Scotland) was washed eight times by vortexing with 0.1 N NaOH in a 1.5-ml microcentrifuge tube. The suspension was centrifuged (15,000 × *g* for 2 min in a microcentrifuge), and the supernatant was decanted after each wash. Fifty micrograms of subtracter DNA (500 μg ml^{-1}) and 50 μl of 0.1 N NaOH were added to the tube, and the contents were mixed by vortexing for 1 min. The suspension was transferred to a siliconized watchglass in a high-humidity chamber for 18 h, followed by air drying for 2 h. The dried matrix was transferred to a 2-ml microcentrifuge tube and washed by vortexing, centrifuging, and decanting with 1-ml volumes of sterile water. One milliliter of 2 M ethanolamine (pH 9.0) was added to the moist matrix and left for 18 h to inactivate any remaining epoxy groups. The matrix was washed five times with 1-ml volumes of sterile water as described above. Before use, the primed matrix was incubated in a 0.5-ml microcentrifuge tube with 200 μl of high-salt buffer (40% formamide, 0.6 M NaCl, 2 mM EDTA, 10 mM HEPES [*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid, pH 7.0], 0.2% sodium dodecyl sulfate) for 5 h with gentle shaking in a 70°C water bath. The matrix was then washed three times with 1-ml volumes of a low-salt buffer (99%

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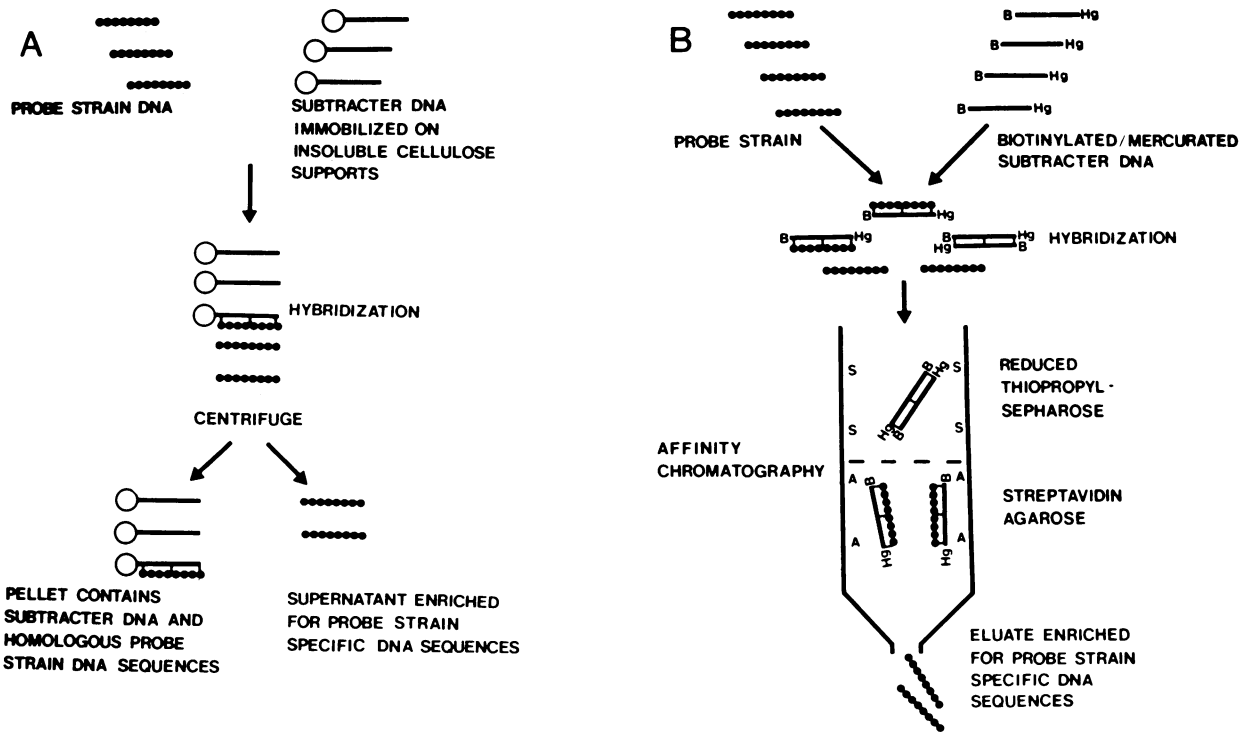

FIG. 1. Schematic representation of heterogeneous (A) and homogeneous (B) subtraction-hybridization procedures.

formamide, 10 mM HEPES), followed by three washes with 1-ml volumes of high-salt buffer with vortexing, centrifuging, and decanting of the supernatant after each wash.

Heterogeneous subtraction hybridization. One microgram of radiolabeled probe strain DNA was hybridized for 18 h at 55°C with 50 mg of the appropriate subtracter DNA-cellulose complex in a 0.5-ml microcentrifuge tube containing 200 μl of the high-salt buffer (in a shaking water bath). Probe strain fragments which had not hybridized to the subtracter matrix were collected by washing the matrix eight times with fresh hybridization solution as described above. The supernatants were pooled and transferred to Centricon 30 microconcentrators (Amicon) and concentrated to ca. 25 μl. The volume was made up to 200 μl with fresh hybridization solution, and the hybridization step was repeated. After each cycle, the cellulose was washed 10 times with 90% formamide–0.2% sodium dodecyl sulfate at 70°C and washed 5 times with hybridization buffer at room temperature. These washes remove any probe strain sequences hybridized to the matrix-bound subtracter DNA, but leave the original subtracter DNA on the matrix (Fig. 1A). The enrichment was performed for a total of five cycles. A probe for strain NZP2037 was obtained by this method, using subtracter DNA from strains NZP2014 and NZP2042.

Biotinylation. A 100-mg sample of sonicated DNA dissolved in deionized water (1 mg ml^{-1}) was mixed with an equal volume of photobiotin acetate (Vector Laboratories) (1 mg ml^{-1} in distilled water) and placed in a sterile plastic petri dish on ice. The mixture was irradiated at a distance of 10 cm with a mercury vapor lamp (Philips, HPLR 400 W), 100 μl of a solution containing 100 mM Tris hydrochloride (pH 9.0)–1.0 mM EDTA was added, and the mixture was transferred to a 1.5-ml microcentrifuge tube. The mixture was extracted twice with 200 μl of 2-butanol to remove excess photolysed photobiotin. DNA was precipitated at −70°C by adding 0.1 volume of 3 M sodium acetate (pH 5.2) and 2 volumes of ice-cold ethanol. The precipitate was collected by centrifuging in a microcentrifuge (15,000 × *g*) for 10 min. The brown-pink pellet was washed with cold 70% ethanol and stored as an ethanol precipitate at −20°C until required.

Mercuration. Biotinylated DNA was suspended in sterile deionized water (100 μg ml^{-1}), and sodium acetate and mercuric acetate (pH 6.0) were added to 55 and 200 mM, respectively. The mixture was incubated at 55°C for 30 h, and the reaction was terminated by adding 0.4 volume of 10 mM Tris hydrochloride (pH 7.6)–1.0 M NaCl–1 mM EDTA. The mixture was dialyzed three times against 1-liter changes of 10 mM Tris (pH 7.6)–20 mM NaCl–1 mM EDTA. The second dialysis solution contained 10 μM 2-mercaptoethanol, which adds a mercury ligand (4). The biotinylated, mercurated DNA was precipitated with ethanol as described above and vacuum dried for 5 min, and the pellet was suspended in 1 mM EDTA.

Preparation of affinity columns. Thiopropyl-Sepharose 6B (Pharmacia) was reduced to the free thiol form by removing the 2-thiopyridyl protecting groups with 0.5 M 2-mercaptoethanol (as described by the manufacturer). Two milliliters of the reduced material was transferred to a glass column (0.5 by 20 cm; Bio-Rad) and allowed to settle under gravity. Excess supernatant was removed, and a layer of siliconized glass wool was placed on top of the settled bed. Two milliliters of streptavidin-agarose (GIBCO Ltd.), which was extensively washed with 0.6 M NaCl and 10 mM HEPES (pH 8), was layered on top of the glass wool. The column was extensively washed with hybridization solution before use.

Homogeneous subtraction hybridization. One microgram of denatured probe strain DNA was mixed with 500 μg of biotinylated, mercurated subtracter DNA in a 0.5-ml microcentrifuge tube. The mixture was adjusted to hybridization

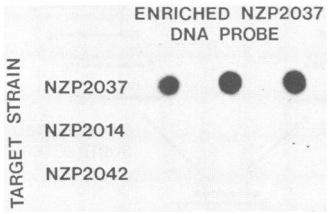

FIG. 2. Hybridization of ^{32}P-labeled *R. loti* NZP2037 probe DNA prepared by heterogeneous subtraction hybridization with triplicate colonies from *R. loti* strains NZP2037, NZP2014, and NZP2042.

conditions (50% formamide, 0.6 M NaCl, 2 mM EDTA, 10 mM HEPES) and hybridized at 35°C in a water bath for 48 h (100 to 130 C_0t). After hybridization, the contents of the tube were diluted with 200 μl of hybridization solution and loaded onto the thiopropyl-Sepharose-streptavidin affinity column. The mobile phase was continuously circulated through the column, using a peristaltic pump at a flow rate of 5 ml h^{-1} for 1 to 4 h or until the counts per minute of the mobile phase remained constant. The column circuit was opened, and unbound DNA was eluted with 5 ml of hybridization solution. The eluate was concentrated to ca. 25 μl using Centricon 30 microconcentrators as described above. The concentrated eluate was mixed with another 500 μg of biotinylated, mercurated subtracter DNA, adjusted to hybridization conditions, hybridized, chromatographed (using a fresh affinity column), and concentrated as above. The affinity materials were carefully removed from the columns and placed in plastic scintillation vial inserts, and the counts per minute were estimated using an LKB scintillation counter. This procedure was used to obtain probes for all three strains (Fig. 1B).

Colony hybridization. Nitrocellulose filters (HAHY; Millipore Corp.) were spotted with triplicate pure cultures of *R. loti* strains NZP2014, NZP2037, and NZP2042 from growth on yeast extract-mannitol agar plates. Colonies were lysed, and the DNA was denatured and fixed to the filters (3). Filters were hybridized at 57°C in a solution of 40% formamide–0.6 M NaCl–2 mM EDTA–10 mM HEPES–100 μg ml^{-1} salmon sperm DNA for 24 h, washed, and subjected to autoradiography (3).

RESULTS AND DISCUSSION

The heterogeneous subtraction hybridization procedure was used to remove sequences from *R. loti* NZP2037 total DNA which are common to strains NZP2014 and NZP2042. After five cycles the enriched NZP2037 probe in the mobile phase was collected and hybridized to colonies of all three strains. Results (Fig. 2) show clearly that cross-hybridizing sequences had been removed to give a probe which yielded a specific signal with the homologous target DNA. With this method of subtraction hybridization, however, a large proportion of the matrix-bound subtracter DNA (ca. 80%) is not available for hybridization with common sequences in probe strain DNA, most likely owing to steric hindrance. Increasing the amount of subtracter DNA from 50 to 500 μg did not improve the method. In addition, after five enrichment cycles with 500 μg of subtracter DNA the remaining mobile-phase sequences cross-reacted with strains NZP2014 and NZP2042 during colony hybridization (data not shown). The failure of this technique with larger amounts of subtracter DNA was attributed to the gradual release of matrix-bound

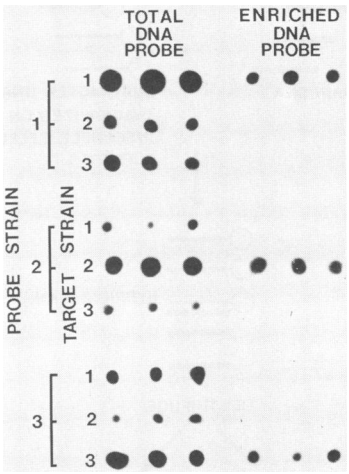

FIG. 3. Hybridization of ^{32}P-labeled total DNA probes and specifically enriched DNA probes with triplicate colonies of *R. loti* strains (1) NZP2014, (2) NZP2037, and (3) NZP2042. For each strain 2 μg of DNA was ^{32}P-labeled as described in Materials and Methods and divided into two equal aliquots. One aliquot was used directly as a total DNA probe (10^6 cpm ml^{-1}). The other aliquot was subjected to homogeneous subtraction hybridization before colony hybridization (10^6 cpm ml^{-1}).

subtracter DNA into the mobile phase (2), caused by the breakage of unstable bonds between certain base positions and the matrix.

Probes for strains NZP2014, NZP2037, and NZP2042 prepared by homogeneous subtraction hybridization demonstrated complete strain specificity when hybridized to target DNA (Fig. 3). With this method, two subtraction hybridization cycles were sufficient to remove cross-hybridizing sequences. Figure 3 also shows the nonspecific properties of total genomic DNA probes from the three strains.

Homogeneous subtraction hybridization was more effective for removing cross-hybridizing sequences. It permitted the use of larger quantities of subtracter DNA, a high proportion of which was available for hybridization since it was not subject to the steric hindrance which affected matrix-bound DNA. Although mercuration and biotinylation reduce the melting temperature of DNA (4, 6), these ligands did not appear to affect hybridization adversely. The use of two independent affinity pairs (biotin-streptavidin and mercury-thiol) ensured the maximum capture of subtracter DNA and any probe strain sequences that hybridized to it. Radioactivity measurements on each component of the affinity capture matrix showed that thiol-Sepharose retained 65% of the total counts per minute.

Although unnecessary for this study, the small amounts of probe DNA generated by subtraction hybridization would normally require amplification to provide a stock of probe for future use. This could be achieved via cloning or by the recently reported polymerase chain reaction (8), which is an enzymatic reaction capable of synthesizing more than a million copies of specific DNA sequences in a few hours in vitro. Labeling of probe DNA need not take place before subtraction hybridization. Amplified sequences could be labeled by incorporating reporter-tagged nucleotides in the final amplification cycle.

The methods reported here were primarily developed to generate probes which would differentiate between a limited number of bacterial strains. One outcome of the study is that interstrain competition experiments for nodulation of *Lotus*

spp. can now be undertaken with any combination of the three *R. loti* strains. The method should also be generally applicable to the isolation of nucleic acid probes from any organism, the specificity of the probe being controlled by the composition of the subtracter DNA.

In conclusion, subtraction hybridization was shown to be a suitable technique for the preparation of strain-specific bacterial DNA probes. The major advantages are (i) the ability to generate specific probes rapidly from organisms for which no previous DNA sequence information is available, and (ii) the elimination of the cloning requirement at all stages of probe preparation.

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